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TITLE: Radiolabeled Herceptin to Increase Treatment Efficacy in
Breast Cancer Patients with Low Tumor HER-2/neu
Expression

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The primary objective of the proposal is to evaluate the efficacy and feasibility of using radiolabeled Herceptin antibody to target rapidly accessible breast carcinoma cells or micrometastases. By using Herceptin to specifically deliver radiation we anticipate that the efficacy of Herceptin will be extended to include breast cancer cells that are not high HER-2/neu antigen expressors. This hypothesis will be tested using the spheroid model to simulate rapidly accessible micrometastases. An alpha-particle emitting radionuclide will be used to enhance tumor cell kill. Completion of tasks 1 and 2 was reported in the previous annual report. Cell kill experiments using Herceptin labeled with an alpha-particle emitter have been carried out; dose-response studies have been completed (task 3). Examination of alpha-emitter and radiosensitizer combinations has begun (task 4: months 21-24) and preliminary work on an animal model to investigate radiolabeled Herceptin targeting of HER2/neu positive disease has been completed (task 5). Results, obtained *in vitro* suggest that alpha-emitter labeled Herceptin will be effective against tumor cells that have a 3 to 4-fold lower expression of HER2/neu.

14. SUBJECT TERMS

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INTRODUCTION

In combination with chemotherapy, the antitumor activity of Herceptin (anti-Her-2/neu), a humanized monoclonal antibody directed against HER-2/neu, has been effective in treatment of breast cancer cells overexpressing HER-2/neu. This promising, FDA approved, and commercially available antibody may be effective in eradicating pre-vascularized micrometastatic disease when labeled with a short lived alpha particle emitter. Alpha particles are very effective in sterilizing cells, and 1 to 3 particles transversing the cell is enough for cell kill. Therefore, this treatment approach may have the potential of eradicating micrometastatic disease both of non-overexpressing and overexpressing breast cancer cells. These hypotheses will be tested first on a tumor spheroid model that can be closely controlled. Spheroids of breast cancer cells expressing different levels of HER-2/neu will be incubated with Herceptin labeled with an alpha particle emitting radionuclide. This model will be used to determine optimal antibody concentration, dose level and treatment schedule. Using the results obtained from the *in vitro* spheroid system, a pilot effort to obtain preliminary data on treatment response *in vivo* will be undertaken. Spheroids will be injected intraperitoneally in athymic mice and response to Bi-213-Herceptin therapy will be monitored using MR imaging. The cells in the injected spheroids will be tagged with a MRI contrast agent. The potential of Dexamethasone to enhance radiosensitivity by increased apoptotic death will be examined. The proposed study may result in a novel treatment approach where Herceptin will be used to eradicate breast cancer micrometastases expressing HER-2/neu.

As noted in the previous annual report, the alpha-particle emitting radionuclide, Bi-213, has been replaced with Ac-225, also an alpha-particle emitter. This was necessary because of limited Bi-213 availability. Ac-225 has a 10-day half-life as compared to 45.6 min. for Bi-213. This makes the labeling and handling of this radionuclide easier, both for experimental work and for eventual clinical implementation. Complete decay of Ac-225 and its daughters yields 4 alpha particles compared to one alpha-particle for Bi-213. Ac-225 labeled Herceptin antibody was obtained from the lab. of David Scheinberg, a collaborator on the grant.

In the previous reporting period, Tasks 1 and 2 were complete in year 1 and work had begun on Task 3 (months 7-20). Task 3 has been completed and makes up the majority of this report. Progress towards development and characterization of an animal model for radiolabeled Herceptin targeting, *in vivo*, (Task 5) is also presented.

BODY

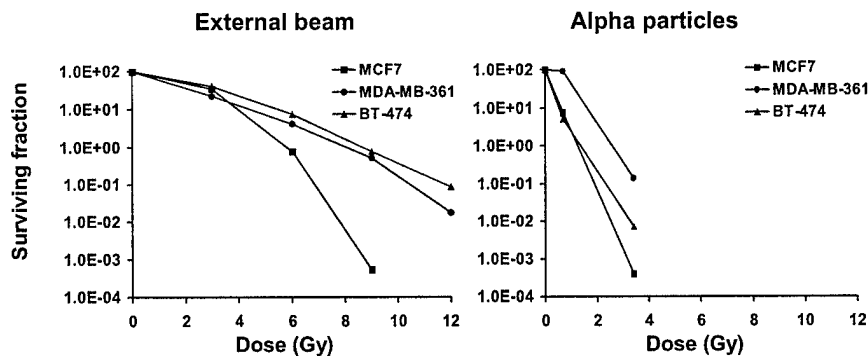
Task 3 (month 7-20) - Perform cell kill experiments using Herceptin labeled with Bi-213. Investigate different Bi-213 activity concentrations and specific activities, relevant and irrelevant antibody. Determine optimal Herceptin concentration and Bi-213 activity for each cell line.

Unlabeled Herceptin dose-response

Results were presented in the previous annual report showing that growth of spheroids of the high, intermediate and low HER2/neu expressing breast carcinoma cell lines, BT-474, MDA-MB-361 and MCF7, respectively, were not affected by a 1 hr incubation with 10 to 500 µg/ml unlabeled Herceptin antibody.

Monolayer culture and spheroid radiosensitivities

To discriminate between inherent radiosensitivity of the different cell lines versus differential expression of HER-2/neu, the radiosensitivity of each cell line to external and to alpha-particle radiation was determined in monolayer culture using the colony formation assay. To facilitate dosimetry calculations (i.e., to control exposure time and geometry) an irrelevant ^{225}Ac -labeled antibody was used in all alpha irradiation experiments. The fraction of plated cells that formed colonies (surviving fraction) vs the delivered (external beam) and estimated (alphas) absorbed dose is plotted below.



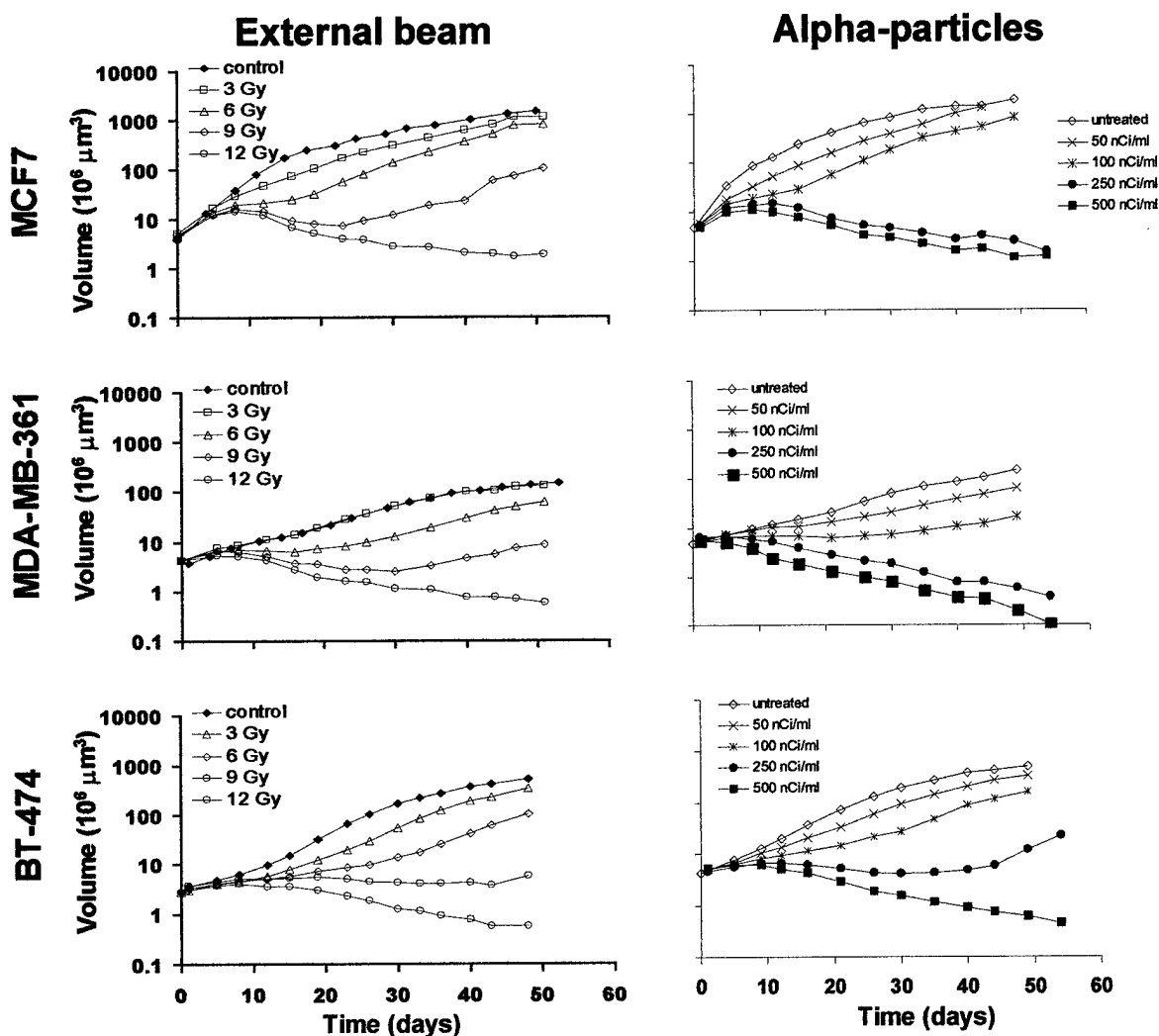
The slope in the log-linear portion of the surviving fraction curves gives an estimate of the radiosensitivity (D_0) of each cell line to the respective radiation. The table below lists the D_0 values (the dose required to reduce surviving fraction to 37%).

Monolayer Radiosensitivity		
Cell line	X- D_0 (Gy)	α - D_0 (Gy)
MCF7	0.6	0.3
MDA MB-361	1	0.5
BT474	1	0.4

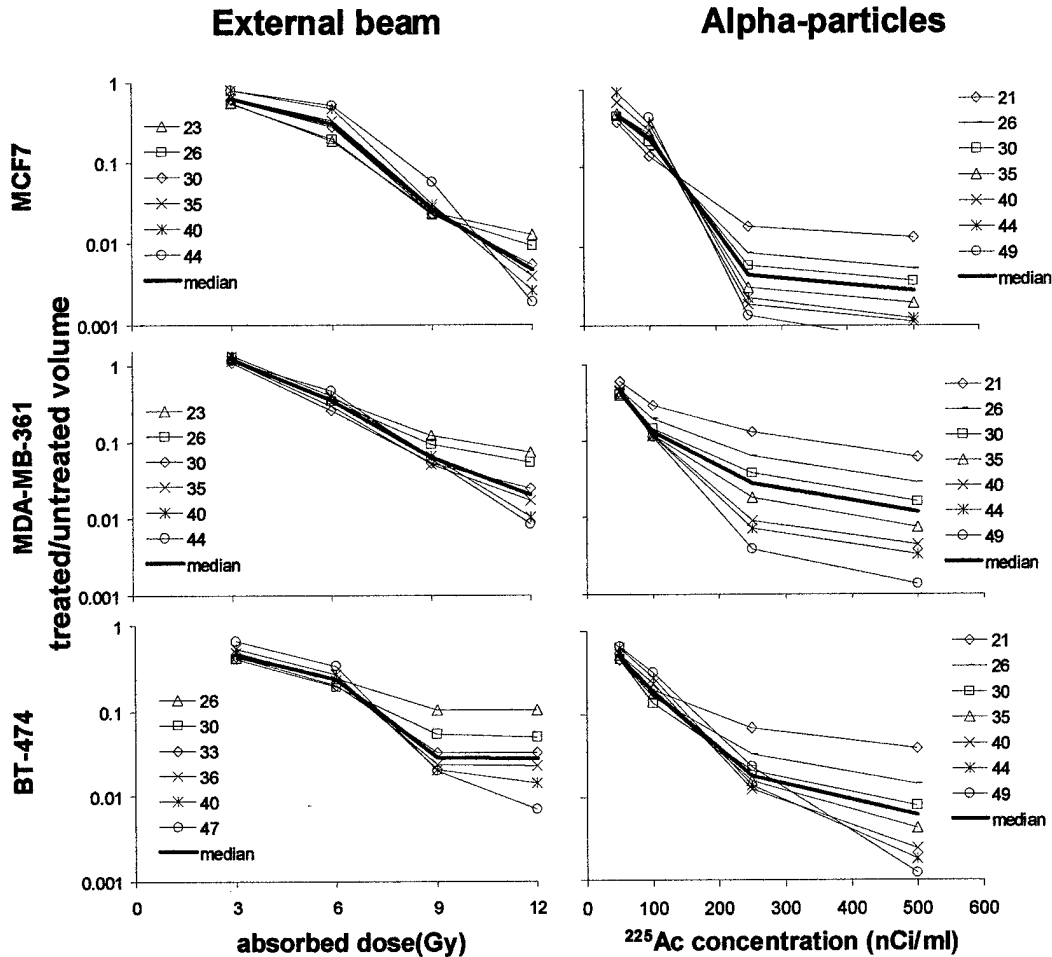
The results show that the two HER-2/neu receptor positive cell lines are approximately equivalent in radiosensitivity, whereas MCF-7, the low HER-2/neu expressing line is more radiosensitive. The difference in alpha-particle radiosensitivity (2nd column of table) is less pronounced than with external beam (1st column).

Spheroid radiosensitivity was assessed for both X-rays and alpha-particles. In both cases, the dose required to reduce the treated to untreated spheroid volume ratio to 0.37 was used as a measure of spheroid radiosensitivity. Since this parameter would depend upon the day post-therapy, a range of days was examined and the median across this range was used. Alpha-particle radiosensitivity was obtained by 24 h incubation with an irrelevant

antibody. Dose-response results are shown below as the median volume of 12 or 24 spheroids versus time post-treatment.



As seen from the control curves, the spheroid growth kinetics of each cell line are different. To estimate radiosensitivities, the results shown above were plotted as the ratio of treated to control spheroid volumes versus dose level for measurements taken over a time period that yields the greatest differences in response (approximately 20 to 45 days post-treatment). The log-linear portion of the resulting curves was fitted to obtain the slope. The inverse of the slope then gives, the dose that yields a treated to untreated spheroid volume ratio of 0.37. This value is denoted, "DVR₃₇" and is analogous to the D₀ value in colony formation assays. This approach makes it possible to obtain radiosensitivity parameters that can be compared across cell lines. The treated to untreated volume ratio figures are shown below.



Each curve in the figure above corresponds to a day post-treatment. The plots provide information regarding the sensitivity of the radiosensitivity parameter to selecting different days for estimating the DVR_{37} . The median curve was generated by taking the median across each day for each dose level. The slope of the log-linear portion of each median curve (i.e., the first 3 points) was then used to estimate the treatment level resulting in a treated to untreated spheroid volume ratio of 0.37. The resulting radiosensitivity parameters are shown in the table below.

Spheroid Radiosensitivities (dose that yields treated to untreated spheroid volume ratio of 0.37, DVR_{37})		
Cell line	external beam (Gy)	alpha-particles (nCi/ml)
MCF7	2	40
MDA-MB-361	2	80
BT-474	2	60

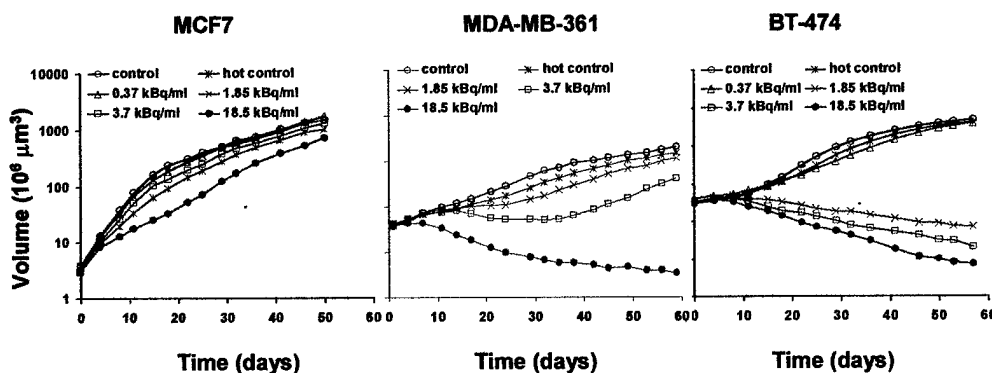
Interestingly, spheroids appear to have a greater differential sensitivity to alpha-particles than to external beam irradiation even though the opposite is true in monolayer culture. In monolayer culture, the intrinsic cellular sensitivity to each type of radiation is

obtained. It is well established that the radiosensitivity of a large variety of cell types falls within a very narrow range whereas radiosensitivity to low LET external beam radiation is known to vary with cell type. The situation in spheroids is fundamentally different, the radiosensitivity parameters for spheroids is not a measure of cell sterilization, but rather of volume reduction. Volume reduction encompasses a large number of biologic variables, including cellular proliferation rate and turnover time.

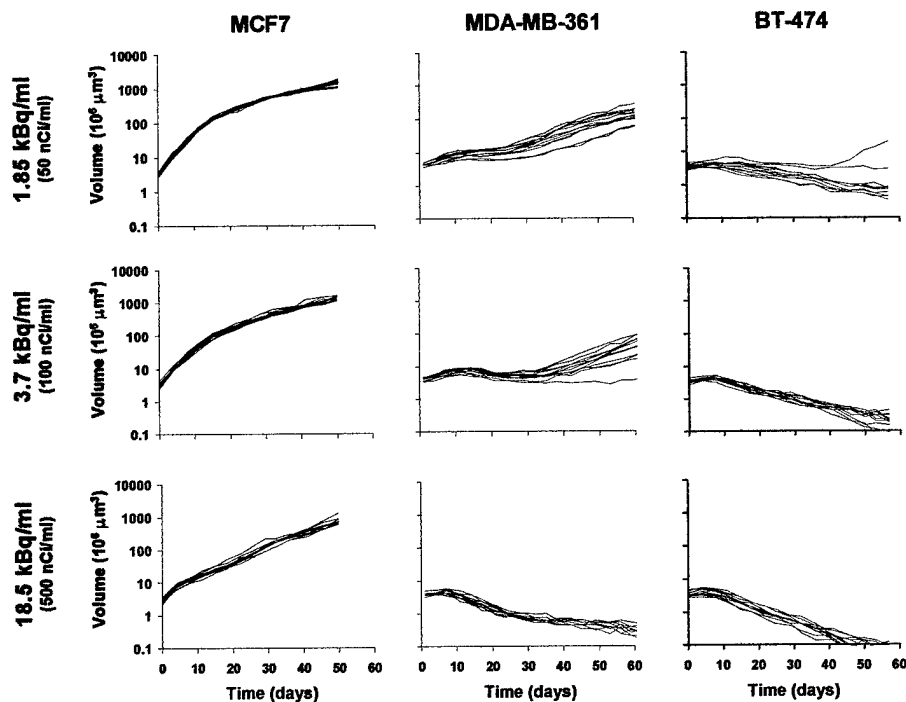
Spheroid kill with radiolabeled Herceptin

The purity of ^{225}Ac -labeled Herceptin was generally $> 90\%$ and the immunoreactivity of the labeled product was between 70 to 80%. Spheroid kill experiments were carried out by incubating 12 to 24 spheroids with Ac-225-labeled Herceptin or non-specific (hot control) antibody in a 2 ml volume; untreated (control) and unlabeled Herceptin antibody (cold control) were also included as controls. Figures 12 through 14 depict results for BT-474, MDA MB-361 and MCF7 spheroids, respectively. The volume, in $\mu\text{m}^3 \times 10^6$ is plotted against time in days; each curve corresponds to the growth history of a single spheroid. All incubations were carried out for 1 hr. Specific and hot control incubations were with 100 nCi/ml Ac-225 on 10 $\mu\text{g}/\text{ml}$ antibody. Unlabeled Herceptin at 10 $\mu\text{g}/\text{ml}$ was used for the cold control experiments. Radiolabeled irrelevant antibody was used for the hot control experiments. Dose-response results are shown in the figures below.

^{225}Ac -Herceptin treatment response

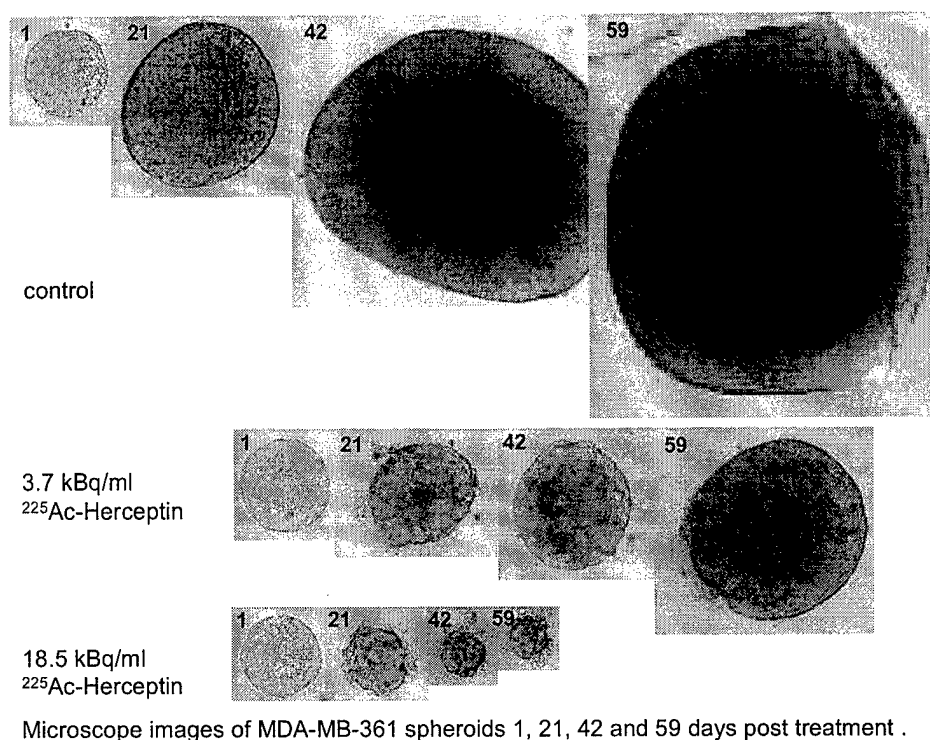


Median growth curves for spheroids incubated 1 hr with 0.37, 1.85, 3.7, and 18.5 kBq/ml ^{225}Ac on 10 $\mu\text{g}/\text{ml}$ Herceptin, or 18.5 kBq/ml on non-specific antibody (hot control).



Growth of individual spheroids following 1 hr incubation with ^{225}Ac -Herceptin.

These results show a strong cytotoxic effect of Ac-225-labeled Herceptin at radioactivity concentrations of 100 nCi/ml for BT474 (high HER-2/neu expressing) spheroids. In contrast, MDA MB-361 spheroids exhibited only a 20-day growth delay following exposure to Ac-225-labeled Herceptin. Corresponding results for MCF7 spheroids showed a very slight effect of Ac-225-Herceptin. Growth monitoring of MCF7 spheroids was stopped at approximately 60 days because the spheroids had become too large for further monitoring by optical microscopy. These figures provide evidence suggesting that the efficacy of radiolabeled herceptin depends upon the antigen expression and that targeting of an intermediate HER2/neu expressor (e.g., MDA-MB-361) is possible. Typical optical microscopy images of MDA MB-361 spheroids at different times (in days) after treatment are shown below.



Microscope images of MDA-MB-361 spheroids 1, 21, 42 and 59 days post treatment .

Task 5 (month 25-26) - Select one cell line and inject spheroids in the peritoneal cavity of mice. Determine baseline growth as measured by MRI.

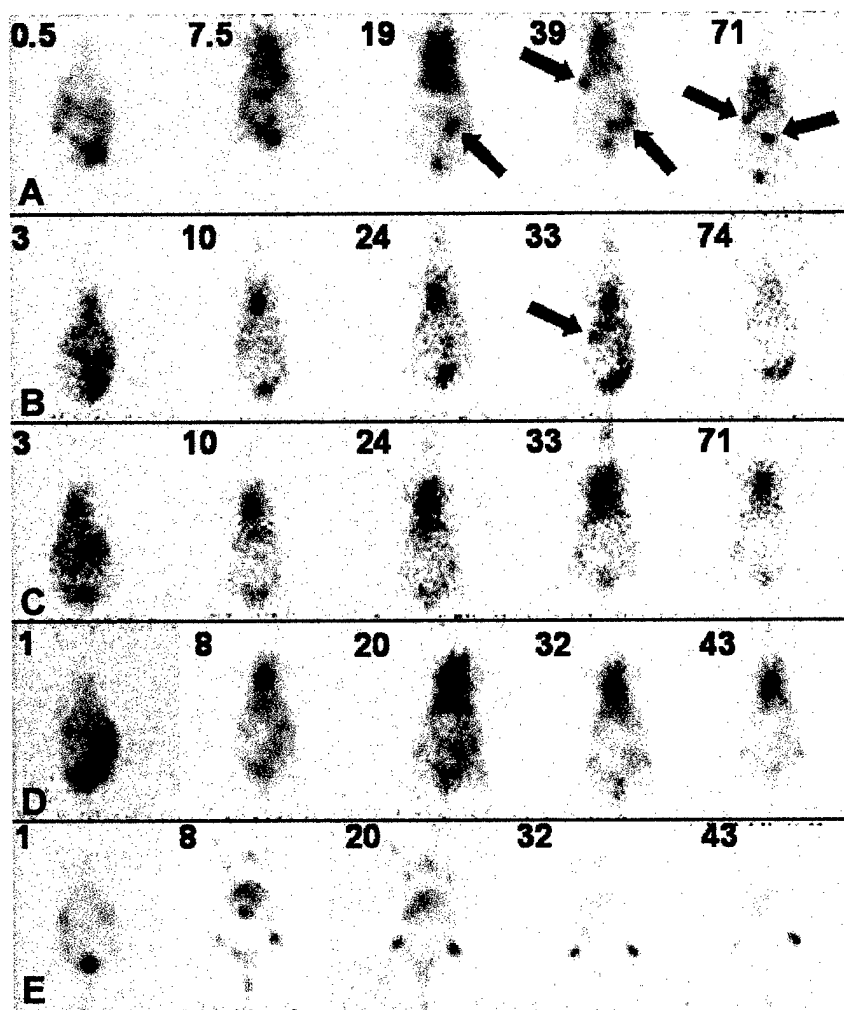
The tumor take following injection of the cell lines listed above was inadequate. We, therefore, went to an ovarian carcinoma model using a cell line with a high HER2/neu expression. A detailed characterization of this model has been carried out. A portion of this work was supported by NIH funds.

MicroPET and MRI-based pre-clinical biodistribution and localization data were obtained using ⁸⁶Y-Herceptin (HER) antibody (anti-HER2/neu) against ovarian carcinoma (1). Female nude mice were inoculated IP with $5 \cdot 10^6$ ovarian carcinoma cells (SKOV-3). 14 days post-inoculation, 12-18 MBq ⁸⁶Y-HER was injected IP (n=8). Tumor-free mice, injected with ⁸⁶Y-HER (n=3), and tumor-bearing mice injected with labeled, irrelevant Ab (n=2), or ⁸⁶Y-HER + 100-fold excess unlabeled HER (n=3) were used as controls. 8 microPET studies per animal were collected over 72-hours. Standard and background images were collected for calibration. MicroPET images were registered with MR images acquired on a 1.5T whole-body MRI. 4.7T small-animal MR images were also obtained for selected time-points. Images were analyzed and registered using software developed in-house. Suspected tumor lesions were dissected for histopathologic confirmation. Blood, excised normal organs and tumor were measured by gamma counting. Uptake was expressed as a localization index (LI \pm SD of the mean): % of injected activity per gram tissue (%IA/g)/ %IA/g in blood. **Results:** IP injection of Ab led to rapid blood pool uptake (5-7 h) followed by tumor localization (15-24 h) as confirmed by registered MR images. Tumor LI was greatest for ⁸⁶Y-HER (7 \pm 1); excess cold HER yielded a 70% reduction. Tumor LI for the irrelevant Ab was 0.9 \pm 0.2. LI for normal organs ranged from 0-2.5 across all studies with maximum LI in spleen. Results are summarized in the figures below. **Conclusion:** For all injected compounds, the relative microPET image intensity of the tumor matched the subsequently determined ⁸⁶Y uptake. Co-registration with

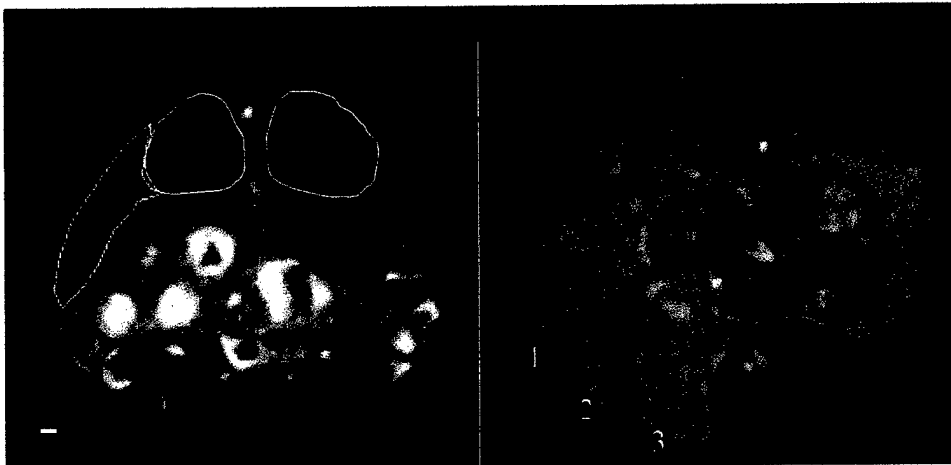
MR images confirmed the position of ^{86}Y uptake relative to various organs. Radiolabeled Herceptin Ab was shown to localize to sites of disease with minimal normal organ uptake.



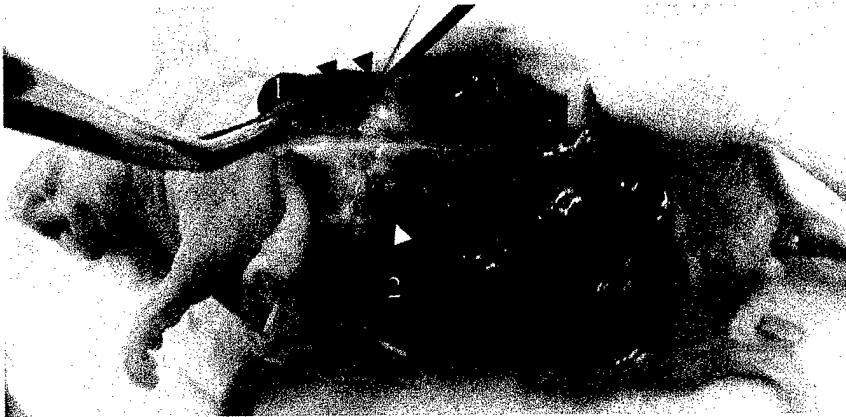
Four transverse microPET image slices showing ^{86}Y -J591 tumor uptake 45h post-injection (top row). Corresponding T2-weighted MR slices showing the tumor as a small 1 mm-diameter lesion at the center of the contours. The spleen is denoted by the dotted blue contour adjacent to the tumor and the two kidneys are marked by "k". Note that there is no kidney uptake of ^{86}Y .



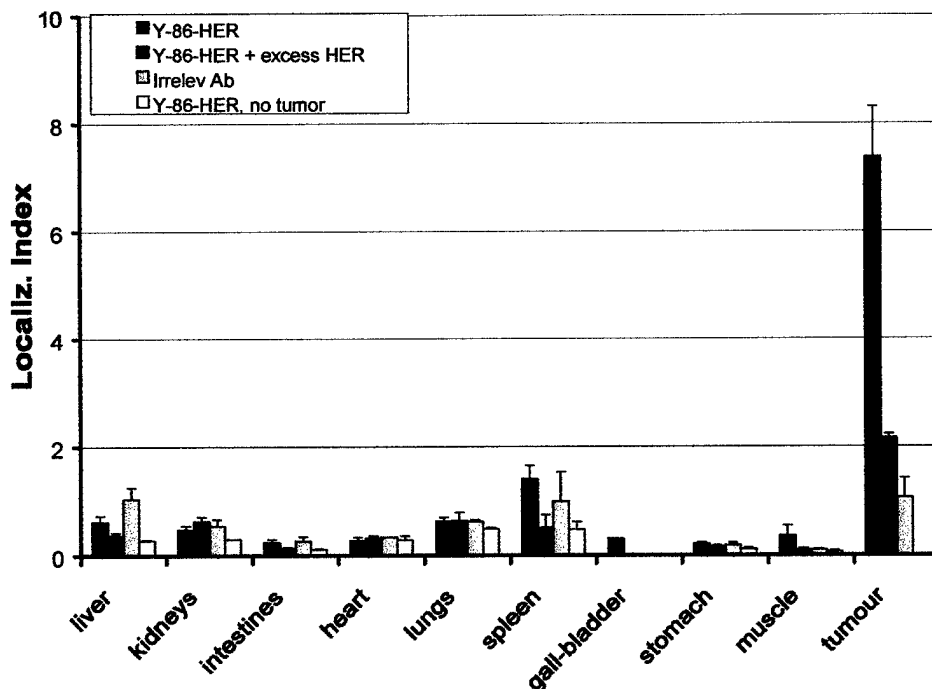
Longitudinal microPET coronal slice images of A: ^{86}Y -Herceptin, B: excess cold Herceptin, C: ^{86}Y -Herceptin in non-tumor bearing mice, D: ^{86}Y -labeled irrelevant antibody and E: free ^{86}Y . Numbers on upper left corner of each panel indicate time post-injection in hours. Arrows indicate antibody accumulation at confirmed tumor sites. All mice show residual activity at the IP injection site. Activity in the circulation is reflected by cardiac activity which is clearly visible in all of radiolabeled antibody images starting at approx. 7 to 10 h post-injection.



Transverse MR image slices of 2 different mice. The left panel shows a slice through the kidneys (light blue), the spleen (yellow) and a collection of tumor nodules (orange). The right panel provides a better depiction of the "beads-on-a-string"-like anatomy of the tumor nodules (contour 1), which may also appear compacted as in contours 2 and 3. The white bar on the lower left of the left panel is 1 mm in length. Both panels are shown on the same scale.



Photograph showing tumor nodules below the spleen (black arrowheads). The spleen (1) is being pushed up by the forceps on the left. The white arrow head points to additional tumor nodules that are just above the liver (2).



^{86}Y -HER biodistribution as measured by gamma counting, post-necropsy. Results are expressed in terms of the localization index (= the tissue to blood concentration ratio).

KEY RESEARCH ACCOMPLISHMENTS

- Generated and characterized the growth of spheroids expressing different levels of HER-2/neu receptor.
- Characterized antibody penetration kinetics into HER-2/neu expressing spheroids.
- Evaluated the external beam and alpha-particle radiosensitivities of three breast cancer cell lines with different levels of HER2/neu expression.
- Evaluated the external beam and alpha-particle radiosensitivities of spheroids of three breast cancer cell lines with different levels of HER2/neu expression.
- Evaluated the response of HER-2/neu expressing spheroids to Herceptin and to Ac-225 labeled antibody.
- Demonstrated feasibility in targeting of intermediate HER2/neu expressing breast cancer spheroids with Ac-225 labeled Herceptin antibody.
- Established and characterized a model that may be used to investigate response to Ac-225-Herceptin therapy.

REPORTABLE OUTCOMES

Abstracts:

Ballangrud AM, Yang WH, Enmon RM, Pellegrini VA, Palm S, Borchardt PE, McDevitt MR, Scheinberg DA, Sgouros G. Alpha-particle emitter (^{225}Ac)-labeled Herceptin antibody targeting of breast cancer: efficacy vs. HER-2/neu expression. Proc AACR '2002; 43:1015.

Palm S, Enmon RM, Xu S, Matei C, Kolbert KS, Borchardt P, Zanzonico PB, Beattie B, Finn RD, Larson SM, Koutcher JA, Sgouros G. Pharmacokinetics of ^{86}Y -Herceptin in an ovarian carcinoma model: correlative microPET and MR imaging. J Nucl Med, 2002; 43:153P.

CONCLUSIONS

We have demonstrated the ability to increase efficacy of Herceptin against tumor cell clusters that have an intermediate level of HER2/neu expression by labeling Herceptin with the alpha-particle emitter ^{225}Ac . The radiosensitivity of cell lines with different levels of HER2/neu expression has been assessed in monolayer culture and as spheroids. The results show that the higher HER2/neu expressing lines were less sensitive to alpha-particle radiation. This observation may have implications regarding the role of HER2/neu expression in radioresistance.

SO WHAT? These studies suggest that by using Herceptin antibody radiolabeled with an alpha-particle emitter it may be possible to treat breast cancer patients whose tumor does not demonstrate high expression of HER-2/neu.